Available online at www.sciencedirect.com
ScienceDirect
Veterinary Parasitology 155 (2008) 190–197
veterinary parasitology
www.elsevier.com/locate/vetpar

Neospora caninum antibodies in wild carnivores from Spain

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Received 11 October 2007; received in revised form 19 April 2008; accepted 6 May 2008

Abstract

Serum samples from 251 wild carnivores from different regions of Spain were tested for antibodies to *Neospora caninum* by the commercial competitive screening enzyme linked immunosorbent assay (c-ELISA) and confirmed by *Neospora* agglutination test (NAT) and/or by indirect fluorescent antibody test (IFAT). Samples with antibodies detected by at least two serological tests were considered seropositive. Antibodies to *N. caninum* were found in 3.2% of 95 red foxes (*Vulpes vulpes*); in 21.4% of 28 wolves (*Canis lupus*); in 12.0% of 25 Iberian lynx (*Lynx pardinus*); in 16.7% of 6 European wildcats (*Felis silvestris*); in 6.4% of 31 Eurasian badgers (*Meles meles*); in 21.4% of 14 stone martens (*Martes foina*); in 66.7% of 3 pine martens (*M. martes*) and in 50% of 2 polecats (*Mustela putorius*). Antibodies to *N. caninum* in common genets (*Genetta genetta*) and Egyptian mongooses (*Herpestes ichneumon*) were only observed by c-ELISA but were not confirmed by IFAT and/or NAT. No antibodies were detected in 5 Eurasian otters (*Lutra lutra*) by any technique. Statistically significant differences were observed among species and among geographical areas. The highest seroprevalence of *N. caninum* infection was observed in the Cantabric Coastal region characterized by high humidity. To our knowledge, this is the first report of antibodies to *N. caninum* in free ranging wild carnivores, other than wild canids, in Europe. The existence of a possible sylvatic cycle could have important implications in both sylvatic and domestic cycles since they might influence the prevalence of infection in cattle farms in those areas.

Keywords: *Neospora caninum*; Seroprevalence; Wildlife carnivores; Spain; Competitive-ELISA; NAT; IFAT

1. Introduction

Neospora caninum is considered as one of the most important causes of abortion in cattle worldwide. It can also cause mortality in other livestock species, companion animals, and in wildlife species. The definitive and

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doi:10.1016/j.vetpar.2008.05.009

intermediate host may be infected by ingestion of water or food contaminated with oocysts, by ingestion of tissue cysts or by transplacental transmission (Dubey et al., 2007).

The dog and the coyote (*Canis latrans*) are the only proven definitive hosts (DH) known to excrete the environmentally resistant oocysts (McAllister et al., 1998a; Gondim et al., 2004a). Many aspects of the life cycle of *N. caninum* are still unknown and the role of wildlife in the life cycle of *N. caninum* is still uncertain. Since the description of coyotes as DH of *N. caninum* (Gondim et al., 2004a) in USA, the role of other wild carnivore species is of interest. Recently, *N. caninum*-like oocysts were found in the feces of free-ranging red foxes (*Vulpes vulpes*) in Canada (Wapenaar et al., 2006) and wolves (*Canis lupus*) are suspected to be a DH for *N. caninum* (Gondim et al., 2004a). A sylvatic cycle involving the white tailed-deer and canids has been proposed in USA; dogs fed naturally infected deer tissues shed *N. caninum* oocysts (Gondim et al., 2004b). However, the importance of similar sylvatic life cycle of *N. caninum* in European ecosystems remains virtually unknown (Hurková and Modrý, 2006).

In Europe, the red fox is the most abundant wild canid and there have been several reports of seroprevalence of *N. caninum* antibodies in this species, e.g.: Austria (Wanha et al., 2005), Belgium (Buxton et al., 1997), Germany (Schares et al., 2001), Hungary (Jakubek et al., 2007), Sweden (Jakubek et al., 2001), the United Kingdom (Barber et al., 1997; Hamilton et al., 2005; Simpson et al., 1997), Ireland (Wolfe et al., 2001; Murphy et al., 2007) and Poland (Smielewska-Los et al., 2003). *N. caninum* DNA has also been demonstrated in brains of red foxes in the Czech Republic (Hurková and Modrý, 2006) and in Catalonia, North-East Spain (Almería et al., 2002), an area where high antibody prevalence (70%) has been recently reported in this canid (Marco et al., 2008).

Little is known of the seroprevalence of *N. caninum* in canids other than the red fox in Europe. One study reported *N. caninum* antibodies in wildlife carnivores in Czech Republic zoos (Sedlák and Bártová, 2006). In Spain, where coyotes are not found, the red fox is the most abundant wild canid species and there are small populations of wolves. However, seroprevalence of *N. caninum* in red foxes in Spain is unknown except for Catalonia (Marco et al., 2008). Recently, antibodies to *N. caninum* in non-carnivore wildlife including red deer, roe deer, barbary sheep, and wild boar have been found (Almería et al., 2007) suggesting the existence of a sylvatic cycle in Spain.

In the present study, serum samples from different areas of Spain were analyzed to investigate seroprevalence of *N. caninum* in wild carnivores.

2. Materials and methods

2.1. Source of animals

Serum samples were collected from 1990 to 2006, from a total of 251 legally obtained wild carnivores belonging to 11 species from five Families (Table 1) surveyed post-mortem (most of them road kills, except fox samples that partly were obtained from hunters) or in vivo (from captures with scientific purposes). With the exception of the red fox, all the sampled species are protected by Spanish law. Sex, age-class and geographic origin were recorded whenever possible. The samples were from six Spanish regions: CC-Cantabric Coastal region, AR-Aragón, CL-Castile and León, SC-Central Spain, CV-Valencia Community, and SH-Seville-Huelva) that are representative of different bioregions of the Iberian Peninsula (Fig. 1). The age class was determined by teeth and other morphological characteristics (Saénz de Buruaga et al., 1991), establishing two age classes: juveniles (under 1 year old) and adults. Blood samples were collected from the heart or chest cavity of dead animals and from the cephalic vein of live animals and sera were stored at −20 °C until analysis was performed.

2.2. Serological examination

Previous studies have shown that one has to be careful interpreting individual data evaluated with only one assay (Pereira-Bueno et al., 2003; Wapenaar et al., 2007a), especially in carnivore wildlife samples (Wapenaar et al., 2007a). Therefore, we analyzed the sera by competitive screening enzyme linked immunosorbent assay (c-ELISA) screening and positive samples were confirmed, when possible, by the *Neospora* agglutination test (NAT) and/or indirect fluorescence antibody test (IFAT).

A commercial competitive ELISA (c-ELISA) from VMRD (USA) was used for detection of *N. caninum* antibodies in wildlife carnivores according to the manufacturers' instructions. Basically, 50 μl of sera samples were incubated in the wells coated with an immunodominant surface protein of 65 kDa specific for *N. caninum*. Another Horseradish Peroxidase (HRP) conjugated monoclonal antibody was added and samples incubated. This monoclonal antibody competes with serum antibodies for a specific epitope on p65.

Table 1
Seroprevalence of *N. caninum* antibodies in wild carnivores from Spain

Family	Species	Total analyzed	Positive c-ELISA (%)	Confirmed by IFAT (positive/tested)	Confirmed by NAT (positive/tested)	Seroprevalence in >1 test (%)[a]
Canidae	Red fox (*Vulpes vulpes*)[b]	95	11 (11.6)	2/9	2/6	3.2[c]
	Wolf (*Canis lupus*)	28	7 (25.0)	0/7	6/7	21.4
Felidae	Iberian lynx (*Lynx pardinus*)[d]	25	5 (20.0)	3/4	0/2	12.0
	European wildcat (*Felis silvestris*)	6	1 (16.7)	0/1	1/1	16.7
Viverridae	Common genet (*Genetta genetta*)	19	1 (5.3)	0/1	0/1	0.0
Herpestidae	Egyptian mongoose (*Herpestes ichneumon*)[e]	23	3 (13.0)	0/2	0/2	0.0
Mustelidae	Stone marten (*Martes foina*)	14	5 (35.7)	1/5	3/5	21.4
	Pine marten (*Martes martes*)	3	3 (100.0)	0/3	2/3	66.7
	Eurasian badger (*Meles meles*)[f]	31	6 (19.3)	0/5	2/5	6.4
	Polecat (*Mustela putorius*)	2	1 (50.0)	0/1	1/1	50.0
	Eurasian otter (*Lutra lutra*)	5	0 (0.0)	0/0	0/0	0.0

[a] Sample was considered positive when more than one test out of 3 was found positive.
[b] There were not available sera from 2 red fox c-ELISA positive samples for IFAT and from 5 red fox c-ELISA positive samples for NAT.
[c] Only 1 of the 3 total samples positive by two techniques was positive simultaneously by NAT and IFAT.
[d] There were not available sera from 1 Iberian lynx c-ELISA positive sample for IFAT and from 3 Iberian lynx c-ELISA positive samples for NAT.
[e] There were not available sera from 1 Egyptian mongoose c-ELISA positive sample for IFAT and for NAT.
[f] There were not available sera from 1 badger c-ELISA positive sample for IFAT and for NAT.

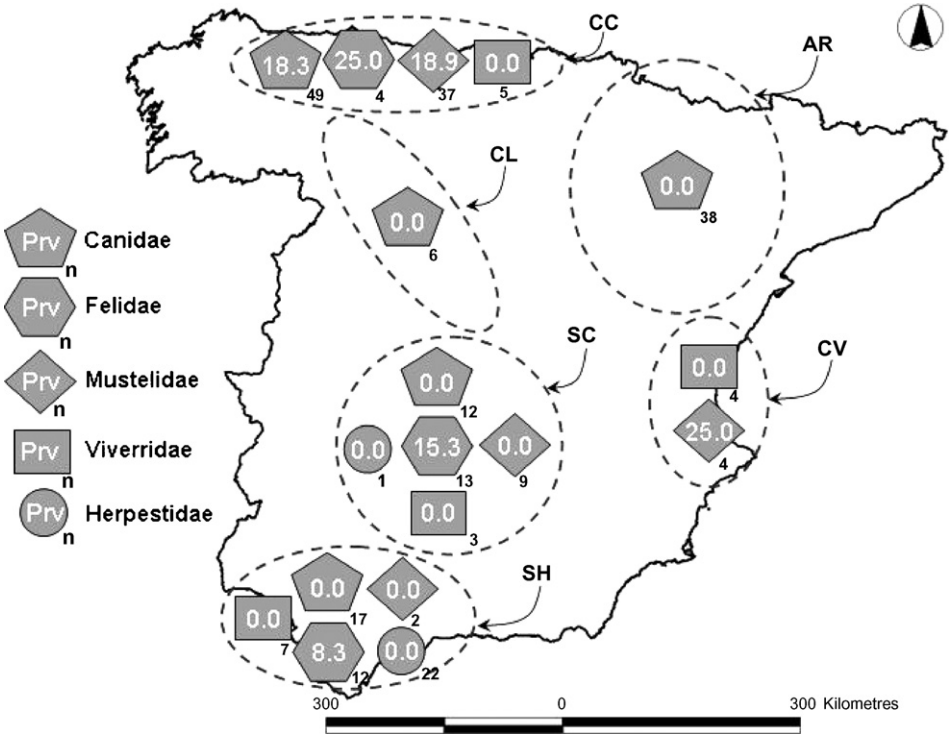

Fig. 1. Map of peninsular Spain showing the prevalence of antibodies against *N. caninum* in five carnivore families (inside the polygons), and the sample size (as subscripts). CC (Cantabric Coastal region); AR (Aragón); CL (Castile and León); SC (Central Spain); CV (Valencia Community), and SH (Seville-Huelva). No location available from 1 sample of the Canidae family, 2 of Felidae family and 3 of Mustelidae family.

After washing, substrate solution was added to the plates, the reaction was stopped and the plates were then read at 620–650 nm absorbance. Positive and negative control samples were provided in the kit. Percentage of inhibition (*%I*) was obtained following the formula:

$$\%I = 100 - \left[\frac{\text{sample OD} \times 100}{\text{mean negative control OD}}\right].$$

If *%I* was more than 40% the sample was considered positive.

The c-ELISA used in this study has been validated for bovine and dog sera (see Dubey et al., 2007). Its principle of competition makes this test theoretically possible to be used in any other species but validation data are not yet available for these species, therefore, confirmation of samples found positive by c-ELISA was sought by NAT at 1:100 sera dilution performed at the Animal Parasitic Diseases Laboratory (APDL) as described by Romand et al. (1998) and/or by IFAT. For IFAT commercially available NC-1 tachyzoite slides (VMRD, Pullman, Washington, USA) and species-specific conjugate were used including rabbit anti-dog IgG (Jackson Immunoresearch Laboratory Inc.) at 1:100 dilution for Canidae and goat anti-cat IgG (Jackson Immunoresearch Laboratory Inc.), also at 1:100 dilution, for Felidae, Musteliade, Viverridae and Herpestidae. *N. caninum* positive and negative dog sera (VMRD) as well as PBS only were used as controls. Only sera giving uniform fluorescence of tachyzoites at antibody titers $\geq$1:20 were considered positive. Sera from positive species screened by IFAT served later as positive and negative controls for such species.

All sera samples were also tested for antibodies to *Toxoplasma gondii* by the modified agglutination test (MAT) (Dubey and Desmonts, 1987) to analyze positivity to both parasites.

2.3. Statistical analysis

Seroprevalence was statistically analyzed considering the variables geographical area, species, sex and age of the animals. The statistical data analysis was performed using the SPSS 14.0 Statistical program. We used nonparametric statistics with 95% confidence level and a p-value <0.05 was considered significant.

Level of agreement between c-ELISA and IFAT was performed using the kappa statistics (Epi Info 6.0) in those species where negative samples were also analyzed. NAT was only performed in c-ELISA seropositive samples and therefore, level of agreement was not performed.

3. Results

Antibodies against *N. caninum* by c-ELISA were detected in 43 (17.1%) of 251 animals. Antibodies by this technique were observed in all the analyzed species with exception of Eurasian otters (*Lutra lutra*) (Table 1) and seroprevalence levels ranged from 5.3% to 100% in the carnivore species where at least one seropositive sample was observed (Table 1).

We confirmed positive samples observed in the c-ELISA test by NAT and/or by IFAT. Due to insufficient volume, not all sera could be confirmed. Of the 43 c-ELISA seropositive sera, 38 were available to be analyzed and confirmed by IFAT (there were no available sera from 2 red foxes, 1 Egyptian mongoose, 1 Iberian lynx and 1 badger), and 33 were available to be analyzed by NAT (there were no available sera from 5 red foxes, 1 Egyptian mongoose, 3 Iberian lynxes and 1 badger). Antibodies considering samples positive to at least two techniques were observed in 21 samples (8.4% prevalence).

The presence of antibodies in at least one sample from red foxes, wolves, European wildcats, badgers, stone martens, pine martens or polecats were confirmed by NAT, while no antibodies were observed in the two available Iberian lynx c-ELISA positive samples by this technique. On the other hand, the presence of antibodies in the Iberian lynx was confirmed in three of four available samples analyzed by IFAT (Table 1), although with low titres. The available c-ELISA positive samples from genet (1 sample) and Egyptian mongoose samples (2 samples) were found negative by both IFAT and NAT and therefore presence of *N. caninum* antibodies in these species could not be confirmed (Table 1). The seroprevalence in red foxes, Iberian lynxes and Egyptian mongooses could have been underestimated because not all c-ELISA seropositive samples were available for confirmation by IFAT and/or NAT.

Statistically significant differences in *N. caninum* seroprevalences were observed among species (Fisher's exact $p = 0.002$), being significantly higher in pine martens than in red foxes ($p < 0.01$), genets ($p < 0.05$), mongooses ($p < 0.01$), badgers ($p < 0.05$) and otters ($p < 0.01$). Seroprevalence was higher in wolves than in red foxes ($p < 0.01$), genets and mongooses ($p < 0.05$) and higher in stone martens compared to red foxes ($p < 0.05$) and mongooses ($p < 0.05$).

Statistically significant differences were observed among regions of origin, with higher seroprevalence of *N. caninum* infection in the Cantabric Coastal region (CC) (17.9%) compared to Aragon (0.0%) ($p = 0.01$) and Seville-Huelva (1.7%) ($p = 0.001$) (Fig. 1). In fact,

17 of the positive samples by at least two techniques (80.95% of the positive samples) were from CC region, which included every positive sample from wolves (6), wildcat (1), red foxes (3), badgers (2), polecat (1) and pine martens (2), and 2 of 3 positive stone martens. Lynxes and Egyptian mongooses are very restricted in location and the samples were all from South Spain (S-H region). However, differences were not statistically significant when seroprevalence by species was analyzed in the different regions, probably due to the small number of samples analyzed in some regions. Differences were not statistically significant among the different species analyzed in the CC region ($p = 0.19$).

All seropositive samples were observed in adults. Antibodies were detected in 12 (8.4%) of 143 adults and 0 (0%) of 31 juveniles. No data were available from 77 samples. However, differences in *N. caninum* seroprevalence between adults and juveniles were not significant.

There were no statistically significant differences in *N. caninum* seroprevalence between sexes (data not shown) or in the seroprevalence of *N. caninum* in red foxes among years of sample collection.

The level of agreement between c-ELISA and IFAT was calculated in red foxes and Iberian lynxes using negative c-ELISA sera as negative controls in IFAT. The kappa value was extremely low (kappa = 0.011) in red foxes but moderate in Iberian lynxes (kappa = 0.694).

All samples used in this survey were tested for both *N. caninum* and *T. gondii*. Higher prevalence of infection by *T. gondii* in the analyzed samples was observed for all the analyzed species. The prevalence against *T. gondii* was of 67.4% of 239 animals analyzed (there were no sera available from 6 red foxes, 1 wolf, 2 badgers, 1 wildcat, 1 genet and 1 Egyptian mongoose). By species, antibodies against *T. gondii* were observed in 54 of 89 (60.7%) red foxes, in 13 of 27 (48.1%) wolves, in 22 of 29 (75.9%) Eurasian badgers, in 3 of 5 (60%) wildcats, in 21 of 25 (84.0%) Iberian lynx, in 13 of 14 (92.8%) stone martens, in 3 of 3 (100%) pine martens, in 5 of 5 Eurasian otters, in 2 of 2 polecats, in 12 of 18 (66.6%) genets, and in 13 of 22 (59.1%) Egyptian mongooses.

4. Discussion

To our knowledge, this is the first large study of seroprevalence of antibodies against *Neospora* in wild carnivores from Spain and the first serologic data for other free-ranging carnivore species, other than wild canids, in Europe. These results indicate that the presence of *N. caninum* infection in wild carnivores could be important in some localized areas of Spain. Care must be taken when comparing these results to studies based on a single test only (e.g. Marco et al., 2008).

Seroprevalence of infection in the red foxes analyzed in the present study considering samples positive to at least two techniques was low (3.2%) but levels could have been underestimated because of the 11 c-ELISA positive samples (11.6% prevalence) only 9 and 6 were available for confirmation by IFAT and NAT, respectively. In addition, most of the samples were collected post-mortem and some were really old and degradation of immunoglobulins could have taken place. The higher detection of antibodies by c-ELISA, especially compared to IFAT, could be due to the antigens utilized by both techniques. While IFAT uses whole tachyzoite antigen, that expose only surface antigens and there is a subjective assessment of the fluorescence, ELISA uses sonicated tachyzoite that could expose both internal and surface antigens. Wapenaar et al. (2007a) observed that IFAT showed an excellent sensitivity with control samples, but the seroprevalence detected in natural samples from red foxes and coyotes was the lowest in comparison with other assays (ELISA, NAT and Immunoblotting). Interestingly, these authors also observed that although seroprevalence levels were in the same range in those species, there was poor test agreement showing that the tests did not classify the same animals as seropositive. Very low agreement in between ELISA and IFAT was observed in red foxes in the present study, although better agreement between both techniques was observed in Iberian lynxes. On the other hand, excellent agreement between ELISA and IFAT was observed in wild ruminants in Spain (Almería et al., 2007) and in general, very good agreement has been observed among different assays for the diagnosis of *N. caninum* in cattle (Von Blumröder et al., 2004; Wapenaar et al., 2007b). These results imply that serological analysis of wild carnivores could be complex and influenced by poor quality of the sera tested.

Wolves have also been hypothesized to be a likely DH for *N. caninum* because wolves are more closely related to dogs that coyotes (Gondim et al., 2004a) or red foxes (Vilà et al., 1999). In the present study, wolves had the highest seroprevalence of infection in the Canidae family (21.4%), being the first report of *N. caninum* infection in this species in Spain and in free-ranging wolves also in Europe. The seroprevalence in wolves in the study was lower than seroprevalence observed by Gondim et al. (2004b) in North America (39% of 164 wolves), but higher than the observed in

Alaskan wolves by Dubey and Thulliez (2005) (3.3% of 122 wolves). The seroprevalence observed in wolves in our study could indicate an important role of this species in the epidemiology of *N. caninum* in the areas where wolves are found in Spain. The differences in *N. caninum* seroprevalence in wolves and red foxes in our study could be related to their diet. While wolf diet is mainly based on ruminants, red foxes are omnivorous.

Silva et al. (2005) observed the same seropositivity to *N. caninum* (8.5% of 59 samples) in captive maned wolves (*Chrysocyon brachyurus*) from Brazil using homologous and heterologous fluorescent conjugates. However, in our study we could not confirm c-ELISA positive samples in European wolves using IFAT with a heterologous conjugate, while NAT showed high agreement. We do not have an explanation for these findings.

In the Felidae family, antibodies were observed in the Iberian lynx (*Lynx pardinus*) and in European wildcats (*Felis silvestris*). Recently, Sedlák and Bártová (2006) observed antibodies in Eurasian lynxes (*Lynx lynx*) in zoos from the Czech Republic. Presence of *N. caninum* antibodies have also been reported in wild feline species such as cheetahs, jaguarondies, Indian lions or lions (Ferroglio et al., 2003; reviewed by Dubey et al., 2007); and antibodies have been observed in naturally infected domestic cats (Dubey et al., 2002; Ferroglio et al., 2005; Bresciani et al., 2007). Felids most probably only act as intermediate hosts in neosporosis. After oral inoculation of cats with tissue cysts of *N. caninum* fecal shedding of oocysts was not observed (McAllister et al., 1998b). So far no clinical cases of *N. caninum* have been described in naturally infected felids, although an experimental study in domestic cats showed *N. caninum* infection in immunocompromised and immunocompetent animals (Dubey et al., 1990). The Iberian lynx is the most endangered species of felids worldwide (Baillie et al., 2004) and some diseases could be serious threats for the conservation efforts of this species (Millán et al., 2007). Further studies should analyze the effect of this parasite on Iberian lynxes.

Although differences were not statistically significant among families, the highest prevalence of *N. caninum* was observed in mustelids (14.5%). This fact has been previously observed by Sedlák and Bártová (2006) in zoo animals from the Czech-Republic analyzed by IFAT, although in different species to those analyzed in our study. On the other hand, DNA of the parasite was not detected in 88 mustelids in the same country by Hurková and Modrý (2006). In America, ermine (*M. erminea*) weasels (*M. frenata*) and ferrets (*M. putorius*) were tested to determine if they could be definitive hosts of *N. caninum* being fed *N. caninum*-infected mice, but oocysts were not observed, so the hypothesis was not supported (McAllister et al., 1999). The main component of these species' diet are small mammals, mainly rodents and lagomorphs, and birds, although they have also been known to eat fruits, invertebrates, carrion and garbage (Prigioni and De Marinis, 1995). In addition, it has been reported that rodents could act as reservoir of the disease by detection of the DNA of the parasite from different tissues and they could play a role in the maintenance and extension of the disease (Ferroglio et al., 2007) and the exposure to the parasite has been detected in brown hare, which could act as a source of infection as well (Ferroglio and Trisciuoglio, 2003). Thus, this diet may allow contact with *N. caninum* more frequently than in other species.

We did not observe antibodies against *N. caninum* in Eurasian otters. Similarly, Gaydos et al. (2007) did not observe the presence of antibodies in 40 marine-foraging river otters (*Lontra canadensis*). *Toxoplasma gondi* infection in the same otters was found to be high (Sobrino et al., 2007). These results could indicate higher water contamination by *T. gondii* oocysts than by *N. caninum* in those areas.

All analyzed species in the present study had higher seroprevalence of *T. gondii* infection (Sobrino et al., 2007) than that of *N. caninum* as has been observed in most of the studies that compare seroprevalence in both parasites (Sedlák and Bártová, 2006; Murphy et al., 2007; Jakubek et al., 2007; Hamilton et al., 2005 among others) indicating that the analyzed animals had more exposure in the natural environment to *T. gondii* than to *N. caninum*.

Prevalence levels of *N. caninum* antibodies in carnivore wildlife species were related to the geographical areas analyzed as observed in wild ruminants in Spain (Almería et al., 2007). Most of the positive wild carnivore samples were from the Cantabric Coast region in North Spain, an area characterized by high humidity that could favor survival and maintenance of *N. caninum* oocysts in the environment.

The results indicated that *N. caninum* infection in wild carnivores in Spain, as happens with red deer (Almería et al., 2007), is localized to certain areas, where, however, it can be present in moderate to high prevalence.

Differences were not statistically significant by age between 143 adults and 31 juveniles tested, probably because the small size of samples affected the statistical analyses. All *N. caninum* seropositive animals were adults. Higher prevalence of infection in adults has been

reported in captive maned wolves (Vitaliano et al., 2004) and in dogs in Spain (Ortuño et al., 2002). The increased infection levels in older animals are probably due to the fact that adult animals have more exposure to *N. caninum* along their life as occur with wild carnivores seropositive to *T. gondii* (Ryser-Degiorgis et al., 2006; Sobrino et al., 2007) and could indicate horizontal transmission as a main route of infection for carnivore species in Spain.

The serological evidence of *N. caninum* infection in most of the wild carnivore species present in Spain with important prevalence levels in some of these species, such as wolves (as possible DH) or mustelids (as intermediate hosts) could have important implications in both sylvatic cycles and domestic cycles since they might influence the prevalence of infection in cattle farms in shared habitats. In Spain, antibodies against *N. caninum* have so far been detected in wild ruminants, wild boars (Almería et al., 2007) and parasite DNA has been found in red foxes (Almería et al., 2002). The role of other wildlife (i.e.: birds and rodents) in the *Neospora* life cycle needs to be investigated. Wild ruminants probably become infected by *N. caninum* ingesting food or water contaminated by *N. caninum* oocysts excreted by canids in the area and the infection maintained by vertical transmission (Almería et al., 2007). In addition, as in other countries, when wildlife is hunted in Spain, carcasses are usually field dressed and the offal is left behind, being available for scavengers, mainly red foxes, feral and domestic dogs in rural areas, completing the cycle. Together with the present results, a sylvatic cycle in Spain seems probable. Further studies are necessary to confirm the existence of such sylvatic cycle in Europe.

Acknowledgements

This study is a contribution to GC-05-006, ICS, JCCM, and agreements between IREC and Principado de Asturias and Ministerio de Agricultura and OAPN, Spain. This study received partially support from the Spanish CICYT, grants AGL2004-06103, AGL2005-07401 and AGL2007-65521. This study was also partially included in the ‘‘Programa de Actuaciones para la Conservación del Lince en Andalucía II’’. Consejería del Medio Ambiente, Junta de Andalucía. R Sobrino was recipient of a Ph.D. grant from Junta de Comunidades de Castilla La Mancha (JCCM). We acknowledge our colleagues from SERIDA, Asturias, and C. Gerique from Valencia for their contribution to this work.

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